THE PARASITISM OF BOTRYTIS CINEREA.

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(WITH TWO FIGURES)

THE classic works of De Bary (1), Kissling (3), and Marshall Ward (2) were the first to call attention to a mode of parasitism in fungi which had not previously been recognized. These investigations brought out the fact that in certain fungi parasitism is brought about by the secretion of a soluble substance by the mycelium which kills and disintegrates the host tissue at a considerable distance from the filaments, thus affording them practically saprophytic nourishment. This substance was thought by each of these investigators to be of the nature of a soluble ferment or enzyme, possessing the power of dissolving cellulose, whereby the injurious effect. Without detracting from the value of these investigations, it may be said that from more recent works on the subject it is evident that there is still much to be explained in regard to the phenomena which these earlier writers described.

The fungi to be considered in this connection form a closely related group which may be designated as the Botrytis-Sclerotinia type. *Botrytis cinerea* has been chosen as the subject of the present article, but the related *Sclerotinia Libertiana*, the subject of De Bary's work, as well as other forms of Botrytis of the *cinerea* type, come naturally into consideration.[1]

As a saprophyte no mold is more generally prevalent than *B. cinerea*, but to the pathologist this species is of special interest on account of its peculiar relations to the phenomena both of saprophytism and parasitism. It is, in a general sense, an example of the facultative parasite of Van Tieghem and De Bary, or the hemi-parasite of Von Tubeuf; the term *Gelegenheits Parasit* of the latter writer describes it more accurately. Briefly

[1] See no. 13 in the list of literature as to the genetic relation of these forms.

stated, the usual conditions under which this organism may affect living plants are as follows: excessive moisture, stagnant air (these two especially when combined with high temperature), low vitality of the host plant, and upon young or delicate parts of plants. While not covering all cases, the parasitic attacks of Botrytis may almost always be ascribed to one or a combination of these conditions. (A number of typical cases of Botrytis attacks are described or referred to in the writer's previous article.) All degrees of parasitic activity occur under these favorable conditions, from growth upon ripe fruit, where the fungus is scarcely more than a saprophyte, to vigorous development upon live growing tissue.

Generally stated, this species is disseminated by means of its conidia, which germinate upon parts of plants and send germ tubes into the living tissue, where they spread about, causing death and disintegration. Kissling found that, unless germination started with saprophytic nourishment at hand, no infection took place, a peculiarity previously discovered by De Bary in *Sclerotinia Libertiana*. Potter (**8**) found, however, that living tissue could be affected directly with conidia in water. Marshall Ward also found direct infection possible in the form of Botrytis which he investigated. The variation in this respect expressed by these results has been found by the writer to be a constant one. With some material direct infection could be produced in a moist chamber, while at other times such attempts were unsuccessful. In all cases much more active infection took place when saprophytic nourishment was used as a starter. The conclusion therefore seems justified that Botrytis varies in the ability of its conidia to produce directly parasitic germ tubes, but with a general tendency to require a saprophytic start.

After infection has taken place, the affected tissue becomes softened and dead and rapidly disintegrates. In the case of fleshy substances, such as turnips and carrots, it is noticeable that, as long as no other organisms become abundant, no disagreeable odor whatever is produced, even when the tissue is thoroughly permeated by the fungus. In *fig. 1* is shown the

characteristic effect of Botrytis filaments upon vegetable tissue, as seen under the microscope. Here is represented a filament of Botrytis growing in the petiole of a lettuce leaf, a soft, succulent tissue. The effect is seen to be a darkening in color, loss of turgidity, disintegration of protoplasm, a separation of the cells from one another, and their final collapse, the tissue being affected considerably remote from the filaments. The same effect is seen in the vicinity of a germ tube penetrating the surface. To the naked eye affected tissue is found to be softened and disintegrated, having the appearance of being boiled. From the nature of this effect it is evident that such a fungus as this is not in a strict sense a parasite. That is, it does not live directly upon living tissue as, for example, the Uredineae, but rather subsists strictly upon dead and disintegrated plant substance. Its parasitism consists in its ability, limited to the conditions already enumerated and varying greatly in intensity, to secrete a substance which has a toxic effect upon living tissue. The nature of this substance may now be considered. Marshall Ward brings out the idea that the filaments secrete a cellulose-dissolving enzyme, which attacks the cell walls and transforms their substance into available food material for the fungus. Kissling records the same result. De Bary showed the same to be true in the case of the species which he investigated.

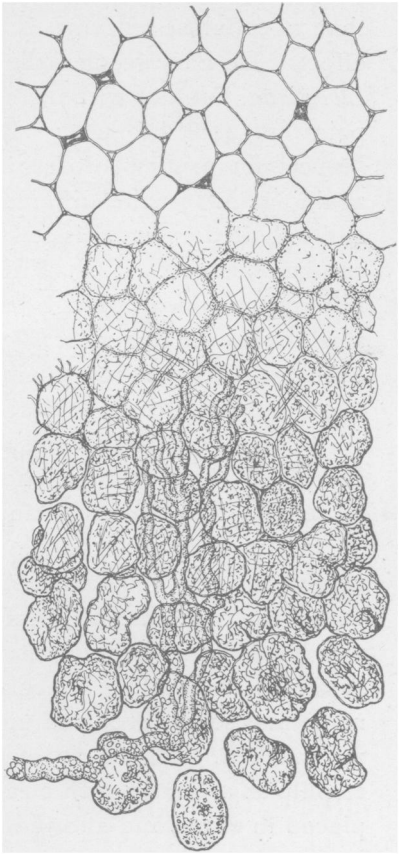

FIG. 1.—Filament of *Botrytis cinerea* invading the tissue of a lettuce petiole.

According to the first author, a watery extract of Botrytis mycelium caused, in thin slices of vegetable tissue, a dissolving of the middle lamellae and strong swelling and final dissolution of the cell walls. When such extract had been previously boiled no effect appeared. No statement is made as to any definite effect upon the cell contents. Kissling also assumes that a cellulose enzyme is the sole cause of the destructive effect. De Bary reaches the same conclusion in regard to *Scl. Libertiana*, but not without some apparent misgivings. At one point (p. 418) he says: "By a single brief boiling the juice [mycelium extract] loses its specific poisonous effect," but again (p. 421) "the difference [between the effects of boiled and unboiled extract] is to be sure a quantitative one so far as can be observed. . . . . With the liquid from sclerotia the same differences appear, though less prominently; the boiled has here a relatively greater effect." Behrens (10) found that boiling an extract of Botrytis mycelium did not destroy its injurious effect upon plant tissue.

It seems reasonable to suppose that a watery extract of the mycelium of this fungus should contain any soluble substances secreted by the filaments, and have the same effect upon tissues, so far as enzymes and any other soluble substances are concerned, as the fungus itself. In preparing such extracts the writer has made use of the following method: Large flasks of any good liquid medium, usually prune juice, were prepared and sterilized, and then inoculated with Botrytis. A vigorous mycelium soon developed upon the surface, which was removed and washed, then cut up into small fragments and finally ground to pulp in a mortar with clean quartz sand. This pulp was then placed in water, and allowed to stand in a warm place for several hours, after which the clearer portion was decanted off, thus getting rid of the sand, and strained. When vegetable tissue was placed in such an extract the effect was very similar to that produced by the growth of the fungus. If a lettuce leaf was placed with the petiole in a flask of the extract all the tissue in contact with the liquid became softened and discolored, and soon

disintegrated, just as in a leaf with the fungus actually growing in the petiole. In a thin section placed in this substance the cells underwent the characteristic changes shown in *fig.* 1, except that more plasmolysis took place. Apparently, therefore, the toxic principle of this fungus is a soluble substance given off by the mycelium.

It was especially noticeable in these tests that the marked swelling of the walls described by Marshall Ward did not occur. No change whatever could be detected in this respect. Behrens and Nordhausen (**12**) also found this to be true with *Botrytis cinerea*, while De Bary's casual mention of a slight swelling can hardly be construed to denote the highly characteristic effect described by Ward. Potter (**9**) found apparently the same effect produced by bacteria. The writer has found no marked change produced by boiling the extract. Certainly the softening of the tissue and death of the cells resulted as before. It is evident, therefore, from the last result alone, that the effects of this fungus upon plant tissue are not entirely due to an enzyme.

The study of the subject has led the writer to the conclusion that two different effects must be clearly distinguished, one following the other: first, the death of the cells; and second, the disintegration of their walls and contents. The further conclusion has been reached that the first effect is produced by a poisonous substance, not an enzyme; the second by a variety of enzymes not necessarily always the same, each affecting its particular substance. The latter conclusion has been deduced from the results of a large number of cultures, made to ascertain the ability of Botrytis to thrive upon various substances of vegetable origin, likely to occur in plants. For this purpose there was first made up a normal mineral-peptone solution, according to one of the usual formulæ, and to portions of this stock solution were added the various substances to be tested, and flask cultures made in the usual manner. These substances may be taken up separately.

Starch.— It was found impossible to grow Botrytis upon this substance, although Behrens states the contrary. In the first

experiments a medium was made up by adding 2^{gm} of commercial cornstarch to each 98^{cc} of the stock solution. The starch did not dissolve to any great extent, but formed a paste. Inoculated with Botrytis only a very feeble growth resulted. From this it seems that this species has not sufficient power of hydrolizing starch into a form of sugar in which it could be assimilated to provide for its full development. It was found, however, that in a very dilute starch solution hydrolysis could be brought about by adding an extract of the Botrytis mycelium, as shown by the iodine test. From this it appears that the fungus secretes at least a small amount of diastase.

Dextrose (glucose, grape sugar).—A 3 per cent. solution of this substance in the stock solution was used as a culture medium. Growth was rapid, and development luxuriant, as was to be expected.

Cane sugar.—A solution containing this substance was made as in the last case. Growth was at first slow, so that at the end of the first week the dextrose cultures were much ahead. Gradually, however, the development became more vigorous, and the final result was as good as any. This course of development gave reason to suppose that it was first necessary to invert the cane sugar before it became available, and that the fungus possessed the power.

Milk sugar (lactose).—The results with this substance were very similar to those with the last; a rather slow start, but eventually a vigorous development.

Maltose.—Vigorous growth from the first.

Levulose (fructose).—Same as last.

Galactose.—Good growth.

Dextrin.—Growth in this substance started rather slowly, but soon became as good as any.

Inulin.—In a 4 per cent. solution of inulin in the stock solution only a very slight growth of Botrytis could be obtained. No normal development whatever took place.

Glycerin.—With 2 per cent. glycerin in the stock solution very good development took place.

Gum arabic.—A similar result to the last was obtained with 4 per cent. gum arabic.

Cellulose.—Cultures were made by using the purest obtainable cellulose, in the form of filter paper, mixed into a pulp with the stock solution. An excellent growth took place in this medium, showing that the fungus was able to utilize cellulose as a food material. Behrens obtained similar results.

Linseed oil.—A 4 per cent. solution of this substance was made in the stock solution. This naturally formed a layer upon the surface, but by frequent shaking the oil was kept mixed with the other liquid for a considerable portion of the time. Growth in such cultures was very good, and a normal development took place. The solution gradually became darker in color until nearly black, while the oil lost its characteristic appearance and the dark colored liquid became homogeneous in appearance.

Cottonseed oil.—Similar results to the last were obtained with this substance.

Tartaric acid.—Two grams of this substance in the dry form were added to 98^{cc} of the stock solution. Growth was excellent throughout.

Malic acid.—With a similar solution of this substance a very vigorous development was obtained.

Oxalic acid.—With this substance in the same proportion no growth whatever appeared.

Formic acid.—To 98 ^{cc} of the stock solution 2 ^{cc} of 98 per cent. formic acid was added. No growth appeared.

Tannin.—A 2 per cent. solution of commercial tannin was made with the stock solution. Growth was excellent, the solution slowly turning black.

Asparagin.—With a 1 per cent. solution of this substance in the stock solution, a very quick growth appeared, developing conidia more quickly than in any other culture. Subsequent growth was not very vigorous.

Salicin.—With a 1 per cent. solution of this substance growth was very slow, but in time reached a fairly vigorous development.

Amygdalin.—In a 2 per cent. solution of amygdalin growth was slow and only fair. The odor of almonds became noticeable in the culture after some development had taken place.

Brucin and strychnin.— In 1 per cent. solutions of these poisonous substances growth was extremely feeble and practically nothing.

Quinin and thein.—In 2 per cent. solutions of these substances no growth took place.

It appears, therefore, from the above results that *Botrytis cinerea* is able to satisfy its carbon requirements from the following substances: sugars in general, dextrin, cellulose, glycerin, gum arabic, vegetable oils, tartaric and malic acids, asparagin, and several glucosides, as tannin, salicin, and amygdalin. The effect of the growth of the fungus upon these substances may now be considered. In studying this point the method used was to reserve a portion of each solution tested, which could later be compared with the remainder of the solution, upon which Botrytis had developed.

SUGARS.

No extensive study was made of the complicated transformations of the various sugars which take place under such circumstances as these. The Fehling test showed that in all cases the solution upon which Botrytis had been growing gave a strong reduction, so that in the case of cane sugar inversion had taken place. It was found that oxalic acid was produced in considerable amount from the sugars, presumably by oxidation, and occasionally traces of acetic acid. Alcoholic fermentation was carefully looked for, but no trace of this substance could be detected in the distillate from the culture fluid. The specific effect of the growth of the fungus upon dextrin, cellulose, glycerin, gum arabic, asparagin, and acids, was not studied. The same is true in regard to the vegetable oils, except that the darkening in color and disappearance of the distinct nature of the oil was apparent to ordinary observation, showing that an important change took place.

TANNIN.

The effect of the growth of Botrytis upon commercial tannin was quite closely followed. It has already been mentioned that the liquid gradually became of a darker color and finally nearly black. The same effect could be shown by mixing a 2 per cent. solution of tannin in a flask of gelatine. A flocculent precipitate occurred, giving the hardened gelatine a white color. When inoculated with Botrytis this color began to disappear about the point of inoculation and turn brown. Gradually the change spread, keeping pace with the growth of the organism, until finally the whole mass of gelatine to a considerable depth had this dark color. This is an excellent method for showing this reaction.

A solution of tannin in the stock solution was divided into two portions and one inoculated with Botrytis. After the end of one week the latter was dark brown in color, while the original portion was still nearly colorless as at first. Both gave a deep purple precipitate with ferric chloride. With calcium hydrate the original solution gave a white precipitate, changing to lilac (tannin reaction.) That upon which Botrytis had grown gave a heavy precipitate, the color of which could not be clearly distinguished on account of the dark color of the liquid. Another portion of the Botrytis liquid was agitated with ether, and the ether then poured off and tested with calcium hydrate. This gave a brown precipitate rapidly darkening (gallic acid reaction). A solution of gelatine gave with the original tannin solution a heavy white precipitate. With the culture fluid no precipitate appeared. After adding an excess of gelatine to both solutions they were again tested with ferric chloride. The original liquid gave no reaction, all the tannin having been precipitated. The other still gave the deep lilac reaction. It was therefore shown plainly that the tannin had been decomposed by the fungus, and that gallic acid was one of the products of decomposition. To portions of each liquid calcium hydrate was added till no further precipitate occurred. These were filtered, and portions of the filtrates tested with ferric chloride until solutions were obtained

entirely free from tannin and gallic acid. With the original solution the final filtrate was perfectly clear. The culture liquid still retained its dark color. These filtrates were then tested with Fehling's solution for sugar. The first gave no result. (It was necessary to remove the tannin, as the substance reduces copper.) The culture fluid gave a strong glucose reaction. These results show therefore that Botrytis decomposes commercial tannin into glucose, gallic acid, and a dark coloring matter whose nature was not further investigated.

SALICIN.

The culture fluid containing this substance was tested with ferric chloride for saligenin, which was always found to be present, though in small amounts. It has previously been mentioned that growth upon this substance was very slow. Apparently the usual decomposition of salicin into saligenin and glucose is effected by Botrytis.

AMYGDALIN.

It has been mentioned that after the growth of the fungus upon this substance was under way, an odor of almonds could be easily detected, which was not apparent in the original solution. This indicates the decomposition of amygdalin into glucose and hydrocyanic acid.

It may be assumed in a general way that these changes in the nutrient media are brought about by the corresponding enzymes, which are secreted by the fungus. It has not been considered necessary to the present discussion to attempt to isolate or study these individually, though this organism is well adapted to such a study. The method of treating substances to be tested with an extract of the mycelium is to be used as a basis for such work. (The citations in Green's work, especially those on the work of Bourquelot and his associates, will be found instructive.) The results of the cultures show in general that this fungus is able to avail itself of most of the ordinary constituents of vegetable tissues when unprotected by vital activity.

The cellulose enzyme calls for especial mention on account of the prominence which has been given to it. It appears to the writer that the discrepancy between the results of Marshall Ward and those of Behrens, Nordhausen, and himself in this respect is to be explained by the varying composition of the substance broadly called cellulose, and the varying ability of fungi to dissolve this substance. In all cases it appears that the more easily affected forms, the hemi-celluloses and pektoses, forming the middle lamellae, etc., were dissolved. That the fungus studied by the writer is able to affect, to some extent, true cellulose, is evident from the cultures on filter paper; but in the case described by Ward, and also that by Potter, where a strong swelling of the cell wall was produced, the most rational explanation seems to be that an entirely different enzyme was present. (See Newcombe (**11**) and Green (**14**), p. 84, on the general subject of cellulose enzymes.) But, however this may be, the point seems clear that the cellulose enzyme or enzymes are subordinate in effect to some toxic substance of a different nature. The effect of the boiled mycelium extract, plasmolyzing and killing the cells with which it comes into contact, is enough to show this. Furthermore, the cellulose enzyme idea of Ward and Kissling allows no explanation of the first entrance of the germ tube through the cuticle. The former says (p. 354) "the tips of the germ-hyphæ attached themselves to the surface of the cuticle, and then *dissolved* their way in, discoloring and destroying the cell walls and cuticle in the immediate neighborhood." It would plainly be impossible for the same enzyme to attack both cellulose and cutin. Again it may be stated, that the *death* of the affected tissue is distinctly a different phenomenon from its utilization by the fungus as food. What then occurs?

There can be no reasonable doubt that some soluble substance produced by the fungus diffuses through the cuticle and cell walls and kills the cells some distance ahead of the filaments. Following this, the filaments, probably under the influence of chemotropism, invade the lifeless tissue in all directions, mostly

by simple mechanical pressure aided by cellulose or pektose-dissolving enzymes. It has not been the writer's observation, however, that the filaments penetrate to the interior of the cells to any great extent. Occasionally a case is seen as shown in *fig. 2*, but here there appears to be no dissolving or swelling of the wall, but a breaking through by simple mechanical pressure. The various other enzymes then become active and the tissue is completely destroyed.

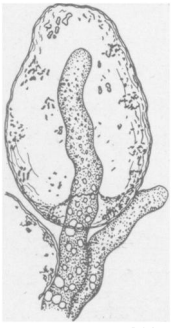

FIG. 2.—Filament of *Botrytis cinerea* penetrating a cell.

The question still remains as to the nature of the poisonous substance. It has already been mentioned, and is a well known fact, that the formation of oxalic acid almost invariably accompanies the growth of Botrytis, being brought about by the oxidation of carbohydrates. Analysis of the mycelium extract also shows this substance in considerable amount. DeBary considered the possibility of this substance being concerned in the effect of the fungus upon vegetable tissue, but discarded the idea on the ground that solutions of pure oxalic acid do not give the entire characteristic effect of a Botrytis extract or culture. The constant occurrence of this substance has seemed to the writer at least suggestive, and its effect upon plant tissues has been studied to some extent. It is of course well known that oxalic acid causes plasmolysis and death of cells if sufficiently strong.

Lettuce leaves were placed by the writer with the petioles submerged in Botrytis extract, and 0.125 per cent., 0.5 per cent., and 1 per cent. oxalic acid. After a few hours of this treatment a remarkably similar effect was observed. All showed marked softening of the tissue where touched by the liquid, followed by collapse and shriveling. The effect of the weakest solution of the acid, 0.125 per cent., was as marked as that of the extract. The boiled extract showed no appreciable difference in effect to the eye from the unboiled. In all five cases the affected tissue had exactly the appearance of having been boiled. The chief

difference in appearance was that the acid had a bleaching effect upon the tissue, while that in the extract became darker colored. Microscopically some similarities and some differences were noted. The cells in the acid were strongly plasmolyzed and killed. In collapsing they pulled apart from one another, so that even in the 0.125 per cent. solution the tissue was almost as completely macerated as in the Botrytis extract. It may be stated conservatively that in a 0.125 per cent. solution of oxalic acid the death of the cells and consequent softening of the tissue was fully as marked as in an average Botrytis extract. The cell wall was somewhat swollen in the acid, but not in the extract. The disintegration of the protoplasm was more marked in the extract. De Bary found in a sample of Sclerotinia extract 0.319 per cent. oxalic acid. The writer has found over 2 per cent. in the mycelium extracts from old cultures to which sugar had been abundantly supplied. It can therefore scarcely be doubted that in such an extract the acid alone would have a marked effect upon plant tissue, whether or not the liquid had been boiled. It is, however, a reasonable objection to judging the effects of the growth of the fungus by those of such extracts, that with germinating conidia or filaments rapidly advancing into new tissue no such amounts of oxalic acid could be expected to accumulate. It is therefore necessary to consider the effects of much more dilute solutions.

Solutions of 0.01 and 0.05 per cent. were prepared, and their effects upon vegetable tissue studied as before. When thin sections of lettuce petioles were placed in these weak solutions, together with others in pure water, the effect was still strongly marked. Within a few minutes a bleaching was evident, and soon the green color had entirely disappeared. Plasmolysis did not occur, but an effect almost identical with that shown in the most newly affected tissue in *fig. 1* soon developed. Especially noticeable is the granular appearance of the affected cell contents, particularly near the walls. Whether this is due to the deposit of an insoluble oxalate, or to a change in the protoplasm, is difficult to determine. However this may be, it appears that

the cells are killed by poisoning, without plasmolysis, both by the fungus itself and by very delicate solutions of oxalic acid, while with the stronger solutions obtained in mycelium extracts or made up directly with the acid the same effect is accompanied by plasmolysis. The softening and bleaching effect of the acid even in 0.01 per cent. solution was very evident to the naked eye.

The production of gallic acid from tannin by the growth of Botrytis has been described above. In a 1 per cent. solution of this substance a lettuce leaf petiole became softened and collapsed much as in the Botrytis extract, but more slowly. A dark color was produced much as in the extract, rather than the bleaching by oxalic acid.

It seems to the writer that we have to seek in some such explanations as these the cause of the poisoning and death of the cells of tissues attacked by Botrytis. Most of those who have considered the subject before seem to have overlooked the fact previously stated that two distinct effects are brought about by the fungus, and that no one substance can produce both of these. That the first or poisonous substance is not an enzyme is plainly proven. That it is oxalic acid seems more than probable from the regular occurrence of this substance and its described effects. The discoloration of the cell walls which the fungus produces, rather than the bleaching brought about by a solution of oxalic acid, is readily explained by the decomposition of tannin or some similar compound.

There may now be considered briefly, in the light of this theory, the tendency in this species to require saprophytic nourishment preliminary to its parasitism. If spores of Botrytis are sown in water upon living tissue no infection ordinarily results. If a drop of prune juice or any good nutrient be added, infection often takes place, under the conditions previously enumerated. This is explained in a general way by the statement that the fungus acquires *vital energy* or *vigor*, by such nourishment. Expressed more definitely, the idea has been that the production of the cellulose-dissolving enzyme is stimulated by this

means, and that the varying ability of the fungus to infect directly depends upon a varying power of enzyme formation from the reserve material of the conidia. The theory advanced in the present article is easily adapted to the known conditions in this respect. The addition of a nutrient solution would at once bring about the formation of oxalic acid, poisoning the subjacent tissue and permitting the entrance of the hyphae through the dead cuticle and epidermis by mechanical force under the influence of chemotropism. That this effect would be favored by the conditions under which Botrytis attacks living tissue needs no explanation; thickness of cuticle and epidermis, and vital activity of the host being the most potent controlling factors. Without such nourishment the ability of the germ hyphae to enter the plant depends upon the amount of acid formed from the reserve material in the conidia. In the linden and rose diseases studied by the writer (**13**) the conidia formed were found to be of unusually large size. Marshall Ward found in the lily disease, where infection took place as well without saprophytic nourishment as with it, that the conidia were much above the ordinary size, and observation in general shows that where Botrytis grows actively and luxuriantly upon living plants the conidia are very large. Are not these points suggestive of an increased power of infection due to the increased amount of reserve material?

SUMMARY.

Briefly stated, the main point of this article is as follows:

In the best known works upon the parasitism of Botrytis and similar fungi too much importance has been ascribed to a cellulose-dissolving enzyme. Two stages in the process should be clearly distinguished: first, a poisoning and killing of the cells; and second, their disintegration and utilization as food by the fungus. The first effect appears to be produced by a substance which there are strong reasons for supposing to be oxalic acid, formed by the fungus as a by-product of its metabolism. Following this, a number of different enzymes are secreted which digest the various constituents of the tissue. The identity of

these enzymes probably varies somewhat in different cases, and apparently more than one occurs which affects different forms of cellulose. The substance causing a marked swelling of the cell wall in the lily Botrytis, studied by Ward, and the turnip bacterium by Potter, appears to be an enzyme not ordinarily produced by *Botrytis cinerea*.

In conclusion, it may be remarked that these results have a very suggestive bearing upon the parasitism of many other fungi which bring about a rapid destruction of the host tissue.

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